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Distribution of *MdACS3* null alleles in apple (*Malus* × *domestica* Borkh.) and its relevance to the fruit ripening characters

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Expression of *MdACS3a*, one of the ripening-related ACC synthase genes, plays a pivotal role in initiating the burst of ethylene production by *MdACS1* in apple fruit. Although previous studies have demonstrated the presence of *MdACS3a*-null alleles through deficiency of transcription activity or loss of enzyme activity due to amino acid substitution, which may affect the storage properties of certain fruit cultivars, an overall picture of these null alleles in cultivars is still lacking. The present study investigated the distribution of null allelic genes in 103 cultivars and 172 breeding selections by using a simple sequence repeat (SSR) marker linked to them. The results indicated that both allelic genes were widely distributed throughout the examined cultivars and selections, some occurring as the null genotype, either homozygously or heterozygously, with each null allele. The implications of *MdACS3a* distribution results and the influence of its null allelotypes in fruit characters are discussed.

Key Words: *MdACS3*, apple, ethylene, ripening, allelotypes, breeding.

Introduction

In climacteric fruits, the process of ripening is accompanied by a marked increase of respiration, which is preceded by ethylene production (Seymour *et al.* 1993). Since both an ethylene receptor antagonist and RNAi directed against genes linked to ethylene biosynthesis inhibit the ripening process (Dandekar *et al.* 2004, Guis *et al.* 1997, Watkins *et al.* 2000) , it is generally accepted that ethylene production is the primary factor leading to the onset of ripening. The first step is the formation of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, from S-adenosyl-L-methionine (SAM) by ACC synthase (ACS). The second step converts ACC to ethylene through the action of ACC oxidase (ACO). In general, the former is the rate-limiting enzyme for ethylene synthesis (Gorny and Kader 1997, Gussman *et al.* 1993, Yang and Hoffman 1984).

Two systems of ethylene production in higher plants have been proposed (McMurchie *et al.* 1972). System 1, the ethylene auto-inhibitory system, is considered to function during normal vegetable growth and to be responsible for the basal level of ethylene production. System 2 is instrumental in the upsurge of ethylene production during the ripening of climacteric fruit when ethylene is auto-stimulatory (Barry *et al.* 2000, Lelievre *et al.* 1997, Seymour *et al.* 1993). The transition of ethylene production from system 1 to system 2 is considered to be an important step during fruit ripening, and is developmentally regulated (Pech *et al.* 2008). In tomato (*Solanum lycopersicum*), which is the primary model for climacteric fruit ripening, system 1 ethylene production is regulated by the expression of *LeACS1A* and *LeACS6*. During the transition period, *LeACS1A* expression is increased, and this is followed by induction of *LeACS4* and *LeACS2* expression in system 2 (Barry *et al.* 2000, Nakatsuka *et al.* 1998). In the case of apple (*Malus* × *domestica*), though four *MdACS* gene families were reported until present (Wang *et al.* 2009), *MdACS1*, which is predominantly expressed in climacteric fruit, is considered to be involved in system 2 ethylene biosynthesis (Sunako *et al.* 1999) because its expression is enhanced by ethylene. Another ripening fruit-specific *ACS* gene is *MdACS3*, which consists of three subfamily genes (a, b, c) located respective

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loci, but two of them (b, c) possess a transposon-like insertion in their 5′-flanking region, which causes failure of their transcriptions (Wang *et al.* 2009). Therefore, *MdACS3a* is only functional *ACS3* gene in apple. It expresses transiently just before the expression of *MdACS1*, *MdACO1* and other ripening-related genes such as the *β*-polygalacturonase (Wakasa *et al.* 2006) and expansin (Wakasa *et al.* 2003) genes, indicating that the function of *MdACS3a* is pivotal in regulating the transition from system 1 to system 2 ethylene biosynthesis. Similar involvement of at least two *ACS* genes has been reported in the ripening process of *Pyrus* (El-Sharkawy *et al.* 2004, Itai *et al.* 1999, 2000).

We have previously reported the presence of two null *MdACS3a* allelotypes in apple cultivars (Wang *et al.* 2009). The first is *ACS3a-G289V*, which has an amino acid substitution altering glycine (GCT) to valine (TCT) in codon 289 at the active site of MdACS3a, resulting in loss of the enzyme activity. The second null allele is *mdacs3a*, which exhibits an absence of the transcript, indicating that this allelotype is deficient in transcription activity. Rough identification of these allelotypes using a simple sequence repeat (SSR) marker linked to the *MdACS3a* allelotype has revealed that the differences in fruit storage properties among several apple cultivars may be explained by these allelotypes (Wang *et al.* 2009). Therefore, the use of DNA markers for the *MdACS3a* allelotype would be very valuable in apple breeding to select candidates with better storage properties.

Here, we investigated the *MdACS3a* allelotypes of 103 cultivars and 172 selections using DNA markers. The results indicated that two null allelotypes were widely distributed in the examined cultivars and selections. The relationship of these allelotypes to fruit ripening character is discussed.

Materials and Methods

Plant materials

One hundred three cultivars and 172 selections from the Aomori Apple Research Institute breeding program were randomly selected and used in this study. The parentages of the cultivars are listed in Supplemental Table 1. Young expanding leaves of sampled trees were used as the source of genomic DNA, which was extracted as described by previously (Sunako *et al.* 1999). Fruits of some cultivars were collected on the day of commercial harvest from the orchard of the Aomori Apple Research Institute, sliced, and then stored at −80°C until use for RNA extraction.

SSR analysis

Primers MdACS3a-1F2 and -1R for ACS3a were used (Supplemental Table 2). The amplification conditions were as follows: initial denaturation at 94°C for 4 min; 32 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension for 5 min at 72°C. A 2-μl aliquot of the PCR product was added to 4 μl post-PCR labeling reaction buffer [15 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.1 μM TAMRA-ddCTP, 0.006 U μl^{-1} Klenow fragment and 0.0288 U μl^{-1} Thermo Sequenase DNA Polymerase] (Kukita and Hayashi 2002). The mixed solution was incubated at 37°C for 5 min, and then at 57°C for 15 min. The reaction was stopped by addition of 6 μl 20 mM EDTA. Appropriate amounts of labeling products were precipitated with ethanol before loading onto an ABI3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The resulting electrophoretogram was analyzed using GeneScan Analysis Software and Genotyper Software (Applied Biosystem).

Sequencing of MdACS3a allelic genes

The promoter region and the coding region of the MdACS3a allelic genes were amplified with the primers MdACS3a pro-LF/-LR and ACS3a infu-1/infu-2 (Supplemental Table 2). PCR was conducted with iProof DNA polymerase (Bio-rad) under the following conditions: predenaturation at 98°C for 1 min, 35 cycles for denaturation at 98°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were inserted into the pGEM-T easy vector (Promega). At least 8 clones for each apple cultivar were sequenced with the ABI 3500 sequencer, and the sequence data were analyzed with BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The accession numbers (Genbank) are JF833308 and JF833309.

CAPS (cleaved amplified polymorphic sequence)/dCAPS analysis of PCR products

The upstream region of *MdACS3a* was amplified by PCR using the primers MdACS3a-1F and -1R. The PCR products were treated with *Sca* I for 2 h at 37°C and then separated on a 1.5% agarose gel. dCAPS (derived cleaved amplified polymorphic sequence) analysis was performed (Neff *et al.* 1998) as follows. The primers MdACS3a G289V dCAPS-Fw/-Rv were designed using the online software dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) (Neff *et al.* 2002). PCR was carried out for 30 cycles of denaturation at 94°C for 3 min, annealing at 52°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 3 min. The products were digested with *Spe*I for 3 h at 37°C and separated on a 2.5% agarose gel. To detect the transcription of the allele, dCAPS primers MdACS3a RT dCAPS -F/-R BamHI, which can yield products with different sizes from the cDNA and genomic DNA because of inclusion of the intron, were designed. The primers and PCR conditions were the same as those described above. The products were digested with *Bam*HI for 3 h at 30°C and separated on a 2.5% agarose gel. All the primers used are listed in Supplemental Table 2.

RNA extraction from flesh and cDNA synthesis

Total RNA was extracted according to the methods described by (Gasic *et al.* 2004) with some modification. In brief, the ground apple flesh was first washed in washing buffer (Hu *et al.* 2002), and then treated twice with phenol-chloroform-isoamyl alcohol (25 : 24 : 1). RNA was

Table 1. *MdACS3a* allelotypes of 103 apple cultivars. Bar (–) indicates none. Single and double underlined cultivars indicate *MdACS1-2* heterozygous and homozygous cultivars, respectively. The cultivars with gray background indicate the *MdACS3a* null cultivars. Triploidy cultivars possessing 3 allelotypes are shown in the margin.

MdACS3a-1	331	Aori 15, Delicious, Granny Smith, Indo, Kotoku, Richard Delicious, Starking Delicious	Triploid Cultivars 331 + 361 + 333 Mutsu, Fukunishiki, HAC-9, Shizuka 331 + 361 + 353 Santaro				
	353	Himekami, Shinsekai	Idared				
	359	Early Strawberry, Melba	–	–			
	361	Gala, Hanaiwai, Kitaro, Kotaro, Sekaiichi, Orei, Raritan, Redgold	Akagi, Fukutami, Hatsuaki, Kagayaki, Megumi	Kitakami, Worcester Pearmain	–		
MdACS3a-2	333	Akibae, Akita Gold, Akiyo, Amabure, Amanishiki, Ambitious, Aori 13, Cox's Orange pippin, Cripps Pink, Esopus Spitzenburg, Freedom, Fuji, Gold Farm, Golden Melon, Harukougyoku, Hida, Hirosaki Fuji, Hokuto, Hozuri, Iwakami, Jerseymac, Kanki, Kinsei, Kio, Kourin, Kyuto, Mikilife, Oirase, Orin, Ousyu, Romu 50, Romu 16, Sansa, ShinIndo, Shinano Gold, Shinano Red, Toki, Toko, Vista Bella	Himekomachi, Jonathan	Akane, Aori 9, Aori 16, Chinatsu, Julyred, Michinoku	American Summer Pearmain, Golden Delicious, Gunma Meigetsu, Jonagold, Koukou, Mellow, Nebuta, Prima, Priam, Ralls Janet, Slimred, Yoko	Aikanokaori, Alps Otome, Kitanosachi, Menkoihime, Narihoko, OBIR2T47, Rome Beauty, Seimei, Shinano Sweet, Tsugaru	
	335	McIntosh, Priscilla	–	–	–	–	–
	SSR	331	353	359	361	333	335
Allelotype		*MdACS3a-1*				*MdACS3a-2*	

precipitated with isopropanol and washed with 70% ethanol. After resuspension with DEPC-treated water, the RNA solution was then treated with chloroform-isoamyl alcohol (24 : 1) and precipitated with 2.5 volumes of 99% ethanol. Genomic DNA was eliminated from total RNA with a TURBO DNA-free Kit (Ambion). The cDNAs used for RT-PCR were synthesized from 300 ng of total RNA with a SuperScript VILO cDNA Synthesis Kit (Invitrogen) in accordance with the manufacturer's instructions.

Results

SSR marker for MdACS3a allelic genotypes

Our previous study revealed that a dinucleotide 'GA' repeat SSR locating 420 bp upstream of the start codon shows polymorphism among the alleles of *MdACS3a* (Wang *et al.* 2009). Therefore, the lengths of PCR amplicons in cultivars and selections were investigated as possible SSR markers for the allelotypes. A representative electrophoretogram is shown in Supplemental Fig. 1 and the results obtained are listed in Table 1 and Fig. 1. Six kinds of amplified product varying in length from 331 to 361 bp (hereafter expressed as only numbers) were identified among the 275 materials (103 cultivars and 172 selections). The differences in length between respective nucleotide numbers were all multiples of 2 bp, indicating that the length difference was due to dependence on the SSR 'GA' repeat number. Five triploid cultivars, including 'Mutsu', exhibited three amplified products consisting of 331 + 361 + 333 or 331 + 361 + 353 (Table 1 and Supplemental Fig. 1). Another triploid cultivar, 'Jonagold' (333 + 361), showed a double peak-height of 333 compared with the other cultivars, suggesting that two alleles (333 + 361) from maternal parent 'Golden Delicious' and the 333 allele from the paternal parent 'Jonathan' (333 + 353) had been combined. All the genotypes of the cultivars and selections for which the parents were known were not contradictory with the genotypes of the parents identified in this study.

Presence of MdACS3a-1 and MdACS3a -2 allele groups

To clarify the linkage between the SSR polymorphisms and *MdACS3a* allelotypes, the genomic sequence of *MdACS3a* in the cultivars 'Fuji', 'Golden Delicious', 'Koukou', 'Ralls Janet', 'Indo', 'Jonathan', 'McIntosh' and

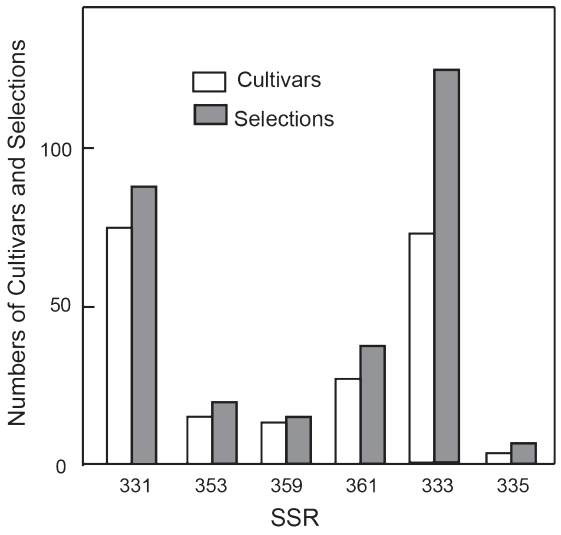

Fig. 1. Numbers of cultivars and selections possessing each of the *MdACS3a* allelotypes. White and grey boxes indicate the data for cultivars and selections, respectively.

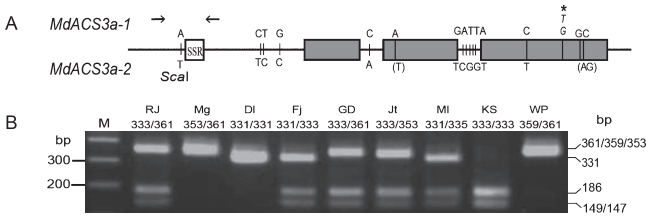

Fig. 2. *MdACS3a* alleles. (A) The SNPs between the alleles are indicated at the upper and lower sides of the schematic structure of *MdACS3a*. The gray boxes show exons and lines between boxes show introns. SNPs T located upstream of SSR in *MdACS3a-2* produces the recognition site of *Sca*I. The primer positions used for identification of the allelotypes are shown as arrows. Asterisked T above the third exon indicates a nucleotide substitution responsible for the *MdACS3a-1V* allele. The nucleotides in parenthesis show that its substitution has led to an amino acid change. (B) Identification of the *MdACS3a* allelotypes using CAPS. The restriction site (*Sca*I) and the primers used are shown in the structure. Only the PCR products from 335/333 allele can be digested into 186 + 149/147 bp fragments by *Sca*I. RJ: Ralls Janet; Mg: Megumi; Dl: Delicious; Fj: Fuji; GD: Golden Delicious; Jt: Jonathan; MI: McIntosh; KS: Kitanosachi; WP: Worcester Pearmain.

'Worcester Pearmain' were investigated. The sequences of all 331 alleles from 'Indo', 'Fuji' and 'McIntosh' were completely identical, then the 331 allele was designated as *MdACS3a-1* (Accession number JF833308). Furthermore, the same *MdACS3a* sequence except for the 'GA' repeat was obtained from the 353 allele of 'Jonathan' and the 359 allele of 'Worcester Pearmain' (Table 1), respectively. Therefore, these alleles were also placed in the *MdACS3a-1* group. On the other hand, the *MdACS3a* sequences of the 361 allele, which were found in 'Ralls Janet', 'Worcester Pearmain', 'Koukou' and 'Golden Delicious', showed only one nucleotide difference from *MdACS3a-1*, leading to our previously reported *MdACS3a-G289V*, in which glycine had been changed to valine at amino acid residue 289 (Wang *et al.* 2009). Therefore, this allele was hereafter designated *MdACS3a-1V*, which also belong to the *MdACS3a-1* group. The *MdACS3a* sequence of the 333 allele of 'Fuji', 'Jonathan', 'Koukou' and 'Ralls Janet', and the 335 allele of 'McIntosh' were identical except for the 'GA' repeat (Accession number JF833309). This sequence was 14 nucleotides different from those of *MdACS3a-1*, which distributed across the whole gene (Fig. 2A). Therefore, we designated them *MdACS3a-2*.

To distinguish *MdACS3a-2* from other alleles, a CAPS was developed. As shown in Fig. 2B, the CAPS DNA marker was able to identify the *MdACS3a-2* genotypes easily.

Non-transcriptional null allelotype, MdACS3a-2

The presence of *mdacs3a*, a null *MdACS3a* allele, was identified because it showed no transcript during the ripening stage (Wang *et al.* 2009). The previous data revealed that the cultivar 'Koukou' was *MdACS3a-G289V* (*MdACS3a-1V* in this report)/*mdacs3a*. Since this cultivar was diagnosed as having a 333 + 361 allelotype, and 361 was one of *MdACS3a-1* (Table 1), the 333 allele identified as *MdACS3a-2* was considered to be *mdacs3a*. To determine whether *MdACS3a-2* lacked transcription ability, we carried out RT-dCAPS analysis to amplify the cDNA fragment of *MdACS3a* with the dCAPS primers (Supplemental Table 2) in the cultivars with the alleles of *MdACS3a-1V/MdACS3a-2*. As shown in Fig. 3A, the shortened fragment (198 bp) after *Spe*I digestion of the amplicon indicated the presence of the *MdACS3a-1V* transcript. The RT-PCR products from 'Golden Delicious' and 'Ralls Janet', both being 333 + 361, were totally digested, indicating that the cDNA originated from only the *MdACS3a-1V* transcript. Therefore, transcription from the 333 allele appeared to be absent. We developed a dCAPS marker in the coding region to distinguish *MdACS3a-1* and *MdACS3a*-2 (Fig. 3B). The cDNAs from cultivars possessing both allelotypes only showed the band corresponding to *MdACS3a-1*, because the PCR products from the *MdACS3a-2* should be cleaved by *Bam*HI. Furthermore, comparative direct sequencing of the PCR products from the genomic DNA and cDNA revealed overlap peaks in gDNA, meaning that *MdACS3a-2* allele in the gDNA were missing in the cDNAs (Supplemental Fig. 3). These results indicate that the *MdACS3a-2* lost their transcriptional activity during fruit ripening.

Discussion

By using SSR and dCAPS markers developed in this work, the features of two *MdACS3a*- null allelotypes, *MdACS3a-1V* and *MdACS3a-2*, which were designated *MdACS3a-G289V* and *mdacs3a* in our previous paper (Wang *et al.* 2009), were investigated in the apple. Although the reason for the lack of transcription from *MdACS3a-2* (333 allele) still remained unclear, there is possibility that three

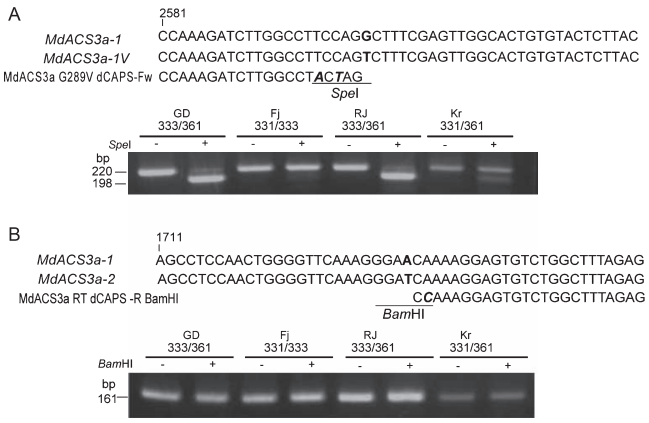

Fig. 3. Confirmation of the *MdACS3a*-null genes by dCAPS markers. (A) dCAPS markers designed for *MdACS3a-1V* (SSR 361). Only the cDNA from SSR 361 is digested by *Spe*I. The results indicate the presence of *MdACS3a-1V* (SSR 361) in 'Golden Delicious', 'Ralls Janet' and 'Kotaro', and the absence of cDNA from the SSR 333 genotype. (B) dCAPS marker to distinguish the cDNAs between *MdACS3a-1* and *MdACS3a-2*. RT-PCR was performed with the primer set MdACS3a RT dCAPS -F/-R BamHI using the total RNAs from mature fruits. Only the products from *MdACS3a-2* can be digested into 134 + 27 bp fragments by *Bam*HI. The lack of the digested product (134 bp) in all the samples means that the cDNA from *MdACS3a-2* (SSR 333) is absent although *MdACS3a-2* allele exists in 'Golden Delicious', 'Fuji' and 'Ralls Janet'. The locations of the primers are indicated by nucleotide position of the *MdACS3a* allele (Genbank Accession number JF833308). GD: Golden Delicious; Fj: Fuji; RJ: Ralls Janet; Kr: Kotaro.

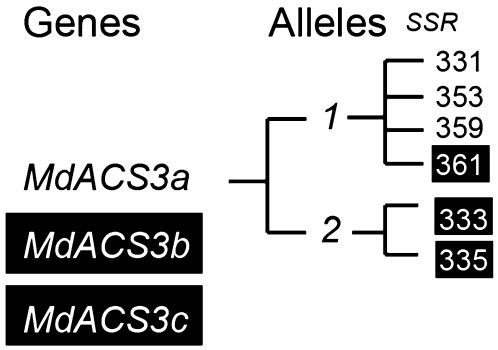

Fig. 4. *MdACS3a* genes in *Malus* × *domestica* and identification by a CAPS marker. (a) Genes in black boxes indicate null alleles. *MdACS3b* and *3c* are unfunctional due to a transposon inserted into the promoter region. The 361 of 3a-1 and 3a-2 (333 + 335) allelotypes are due to an amino acid transition and loss of transcription activity, respectively.

nucleotide substitutions in the promoter region may be responsible, because they locate at -219, -217 and -189 bp from the transcription start site where is normally critical *cis*-element distribution. Furthermore, with regard to the 335 allele of *MdACS3a-2* in 'McIntosh' and its progeny 'Priscilla', its transcriptional activity could not be examined in this study due to the unavailability of ripening fruit at the time. However, as this allelotype has the same sequence as the SSR 333 allelotype except for the length of the SSR, it may also be considered null.

In the cultivars and selections examined in this study, *MdACS3a-2* and *MdACS3a-1V* were distributed in approximately 80% and 24% of them in a heterozygous or homozygous state, respectively. Apple cultivation in Japan originally began using cultivars imported from the USA in the late nineteenth century. Subsequently, some cultivars adapted well to the environment, and were used as elite breeding parents, mainly 'Ralls Janet', 'Golden Delicious', 'Delicious', 'American Summer Pearmain', 'Jonathan' and 'McIntosh' (Kon *et al.* 2000). Surprisingly, the first two cultivars possess null genotypes with a combination of *MdACS3a-2* and *MdACS3a-1V*. Furthermore, 'Jonathan' is heterozygous of *MdACS3a-2*. The finding that the *MdACS3a-2* (333 allele) was the most abundant after the 331 and 361 alleles in the present study materials may be due to the fact that these original cultivars had been used for breeding. On the other hand, each cultivar had been selected by experienced breeders from the respective breeding mass. Since two *MdACS3* subfamily genes, *MdACS3b* and *MdACS3c*, are of the null type because of a transposon inserted in the promoter region (Wang *et al.* 2009), the cultivars and selections homozygous for the *MdACS3a*-null type gene would lose the function of the *MdACS3* gene (Fig. 4). Therefore, the *MdACS3a*-null allelotype likely contributes to the better storability of apple fruit (Wang *et al.* 2009).

Is there any evidence that cultivars homozygous for the *MdACS3a*-null genotypes exhibit a long shelf life? Twelve and ten cultivars were found to have the null allelotype of *MdACS3a-1V*/*MdACS3a-2* and *MdACS3a-2* homozygosity, respectively (Table 1). Given the fact that *MdACS3a* functions initiate a burst of ethylene production by *MdACS1*, the ethylene burst in these cultivars with *MdACS3a*-null genotypes may be influenced. On the other hand, fruit maturation signal(s) and ambient temperature at the onset of ripening are also known to greatly influence ethylene production (Dal Cin *et al.* 2007, Kubo *et al.* 2009). Eventually early- and middle-maturing cultivars may easily enter the system 2 stage, regardless of their *MdACS3a* allelotypes (Wang *et al.* 2007). Therefore, the null genotype is considered to influence the initiation of ripening of only later-maturing cultivars. In our previous studies, some of 22 *MdACS3a*-null allelotype cultivars have been investigated for their internal ethylene concentration in ripening fruit, which is known to parallel the ethylene production rate. Indeed, they reported that the late-harvest cultivars 'Ralls Janet', 'Gunma Meigetsu', 'Slimred' and 'Narihoko' exhibit very low ethylene levels as compared with the late-harvest cultivar 'Fuji' during storage even at 24°C for 12 days (Harada *et al.* 2000, Wakasa *et al.* 2006). Taken together, we concluded that the null *MdACS3a* consisting of two types, alleles of no transcription and of no enzymatic activity, affects the ripening initiation of only late harvest cultivars but not early or middle cultivars.

However, Zhu and Barriff (2008) have reported that

although the influence of *MdACO1* (ACC oxidase) genotypes plays a minor role in comparison with *MdACS1*, the association between the *MdACS1* and *MdACO1* allelotypes is also considered to influence the storability of harvested fruit. Therefore, the *MdACS3a* genotype alone cannot explain the full spectrum of ethylene production by which ripening progression is controlled. Moreover, the ethylene that is synthesized mediates the ripening process via the receptors (Tatsuki 2010), eventually triggering enzyme activity like that involved in the modification of cell walls (Tacken *et al.* 2010, Wakasa, *et al.* 2006). The genes encoding these ripening-related enzymes are probably composed of multiple allelotypes, because of the existence of hemizygous DNA in the heterozygous *Malus* genome (Velasco *et al.* 2010). Although more studies will be needed for a complete understanding of the differences in fruit storage properties among apple cultivars, knowing the distribution of the *MdACS3a* allelotypes in cultivars and selections would be contributory to elucidate the complex ripening molecular mechanism in apple.

Acknowledgments

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